iCyte/iCys Revision 3.0 User Tutorial
Exercise 4

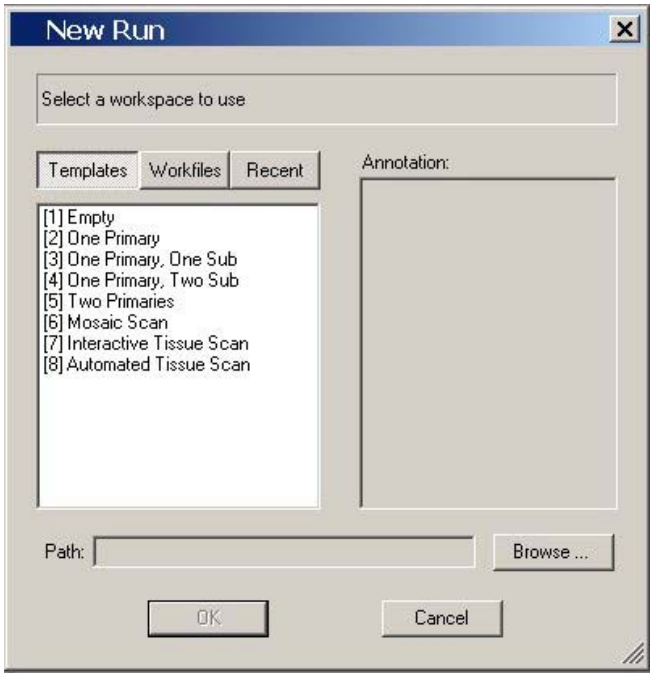
Exercise 4: Multi Scale Scan

The purpose of this exercise is to allow the user to become familiar with setting up multi-scale scans. Multi scale scans provide the iCyte/iCys user with the ability to perform a fast, low resolution scan to identify the area of interest, then will automatically perform a high resolution scan.

The sample used of this exercise is a breast tissue micro array slide. The breast tissue was tested for target antigen HER2/neu, stained with diaminobenzadine (DAB) and counter stained with hematoxylin. Since there is no fluorescence staining, the chromatic absorption technique will be used identify areas of staining.

Start-up/Creating Workspaces

1. Turn on the iCyte main power and laser power.
2. Turn on the computer and all accessories.
3. Launch the iCyte application by double clicking on the iCyte desktop icon. The main iCyte screen will open.
4. From the side Task Bar select **New Run**. The New Run window will open:



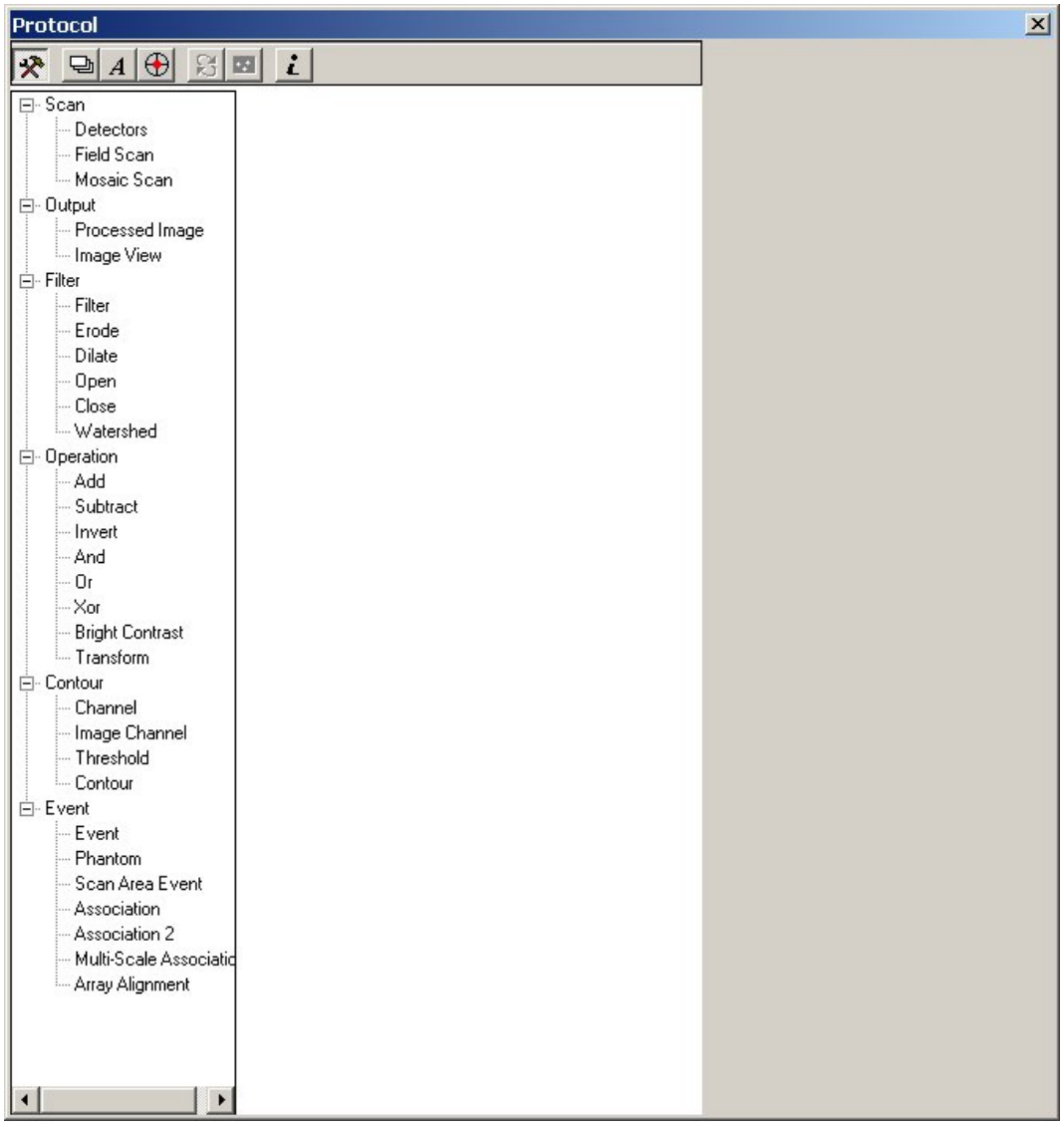
It is from this window that you will begin the process of setting up a workspace for scanning.

CompuCyte has provided a number of Templates that cover the most common scanning modes. These templates can be used as a starting point to build customized Workfiles.

5. For this exercise you will build a template from scratch. Highlight the Templet box and select **Empty** from the Template list and click on **OK**. A single flex-tab titled Protocol and a blank Protocol window will open.

6. Click on the Select Carrier Type icon in the Carrier window and change the carrier to 4 Slide Carrier

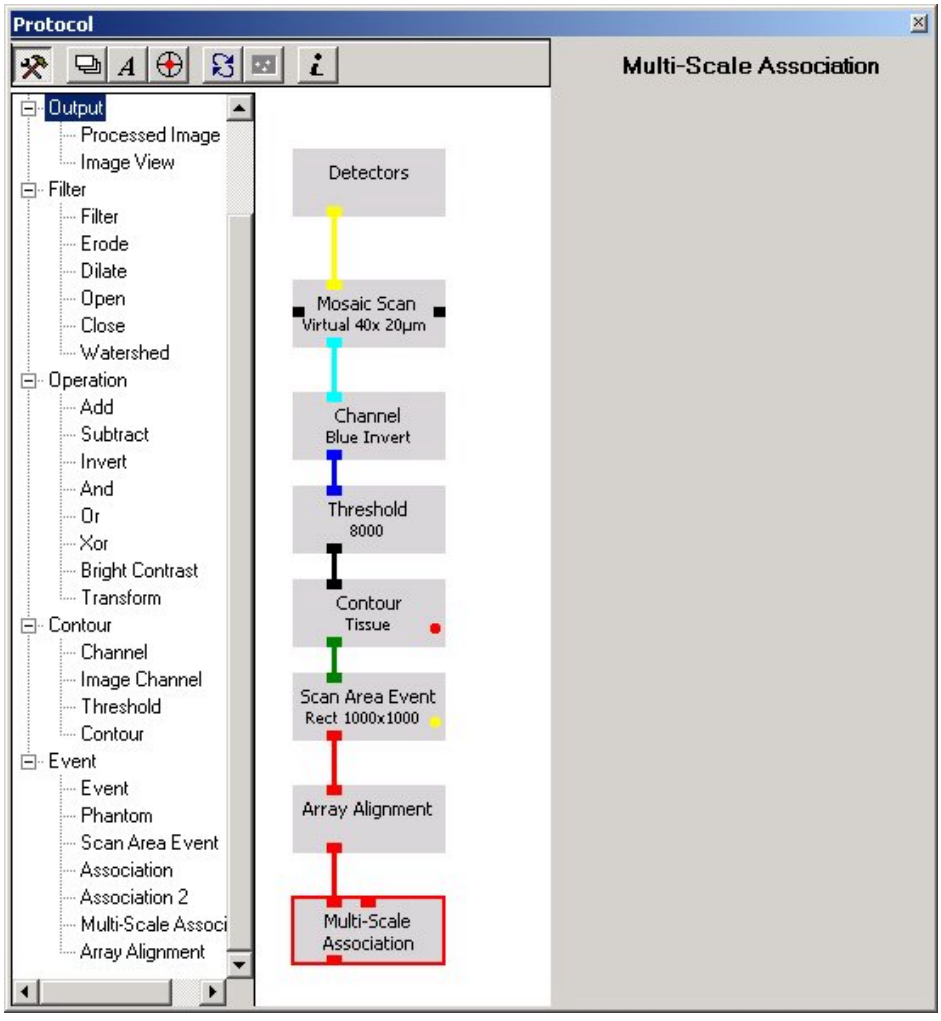
7. Click on the Tool Box icon  in the Protocol window to display the list of Modules



Adding Modules to Protocol

8. The first leg of a multi scale scan is the low resolution (scout scan) at a higher X step value to identify where the tissue sections are located on the slide. In this exercise, you will create a virtual channel using the inverted scatter signal from the argon laser to identify the tissues sections. To create this leg, add the following modules to the Protocol window and connect the modules.

    Detector
    Mosaic Scan
    Channel
    Threshold
    Contour
    Scan Area Event
    Array Alignment
    Multi Scale Association



9. Set up the low resolution scan parameters as follows:

Module	Field	Setting
Detectors	Laser-First Pass	488 nm
	Channels	Scatter - Lightloss
	Virtual Channel Name	Blue Inverted
	First Channel	Scatter
	Operand	Invert
Mosaic Scan	Objective	40x
	X Step size	20 microns
Channel		Blue Invert
Threshold		Manual - 5000
Contour	Component Name	Tissue
	Min Area	3 mm^2
	Color	Red
Scan Area Event	Rectangle	1000 µ x 1000 µ
	Component Name	TMA
	Color	Yellow
	Show Event Name	Checked (on)

Refer to Exercise 1 for information on setting parameters within the modules.

10. Add the following modules to the Protocol window to construction the second scale or high resolution scan

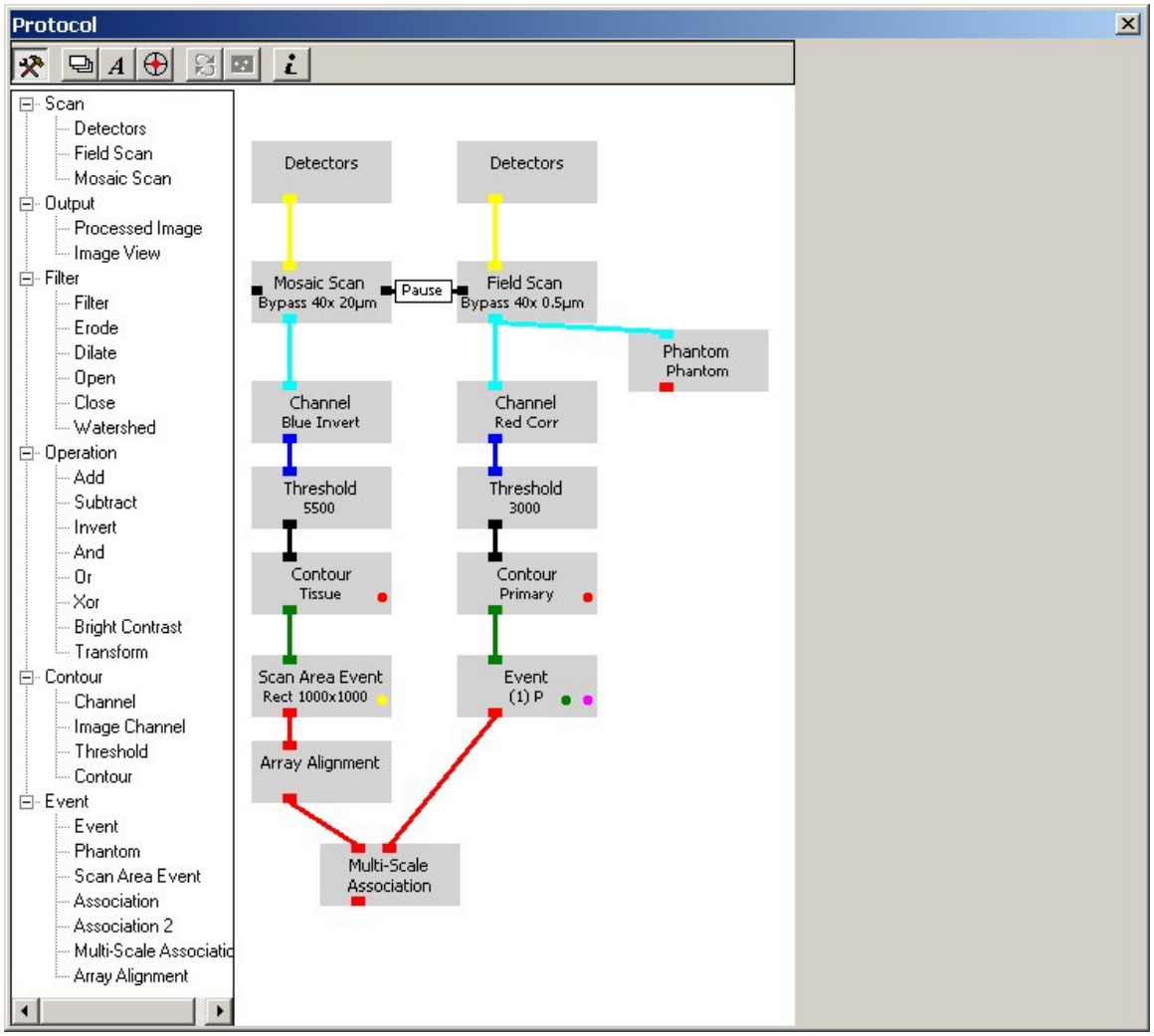
    Detectors
    Field Scan
    Channel
    Phantom
    Threshold
    Contour
    Event

    Connect the modules of the second leg to one another.

    Connect the cyan node of the Phantom module to the cyan node of the Field Scan module.

    Connect the Event module to the right most red node of the Multi-Scale Association module.

    Connect the right most black node of the Mosaic Scan to the black node of the field scan. Click on this link until the word Pause appears. This will stop the scan between the first, low resolution scan and second high resolution scan so that adjustments can be made to the scan areas.



11. Open the Detector module of the second scale and then open the Channel Tab. Set up as follows:

Module	Field	Setting
Detectors	Laser-First Pass	488 nm
	Detector	Green
		Scatter
	Laser-Second Pass	633 nm
	Detector	Scatter2

12. Open the Virtual Channel Tab and create the following channels:

Title	First Channel	Operator	Second Channel/Operand	Purpose
Blue Inv Scat	Scatter	Invert		Intermediate for compensation
Red Inv Scat	Scatter2	Invert		Intermediate for compensation
Red Light loss M	Red Inv Scat	Multiply	0.3	Intermediate for compensation
Blue Corr	Blue Inv Scat	Subtract	Red Light loss M	DAB Quantification
Blue Light Loss M	Blue Inv Scat	Multiply	0.6	Intermediate for compensation
Red Corr	Red Inv Scat	Subtract	Blue Light loss M	Hematoxylin segmentation

12. Continue setting up the scan parameters of the second scale as follows:

Module	Field	Setting
Field Scan (General)	Objective	40x
	Step Size	0.5 µ
Field Scan (Advanced)	Correct Pixel	On
	Field Image	Save - jpeg
	Mosaic Image	Save – Pixel size 5 Sampling: Single
Phantom	Name	Phantom
	Color	Cyan
	Pattern	Random
	Radius	10 µ
	Min. distance between centers	1 µ
	Count per field	1000
Channel	Channel	Red Corr
Threshold	Manual	3000
Contour	Name	Primary
	Min area	10 μ^2

	Color	Red
Event	Contour	Expand by 1
	Color	Green
	Background	Manual
	Color	Blue
	Peripheral	On range 6 to 24
	Color	Magenta

13. Initialize the protocol by clicking on the  in the Protocol Window icon bar.

Data Display

14. Select **Set up** from the top menu bar and **New Tab**.
15. Give the new tab the title **Data** and select **OK**.
16. Select **View** from the top menu bar then New Scattergram, repeat this 5 more times to create a total of 6 scattergrams.
17. Configure the scattergrams as follows:

Scattergram	Component	X Axis	Y axis
1	Primary	X position	Y position
2	Phantom	X position	Y position
3	Primary	Red Corr Integral	Blue Corr Peripheral Integral
4	Phantom	Red Corr Integral	Green Integral
5	Phantom	Red Corr Integral	Blue Corr Integral
6	Phantom	Red Corr Integral	Blue Corr Integral

18. Select View from the top menu bar and select New Histogram.
19. Configure the histograms as follows:

Histogram	Component	X Axis
7	Phantom	Blue Corr Integral

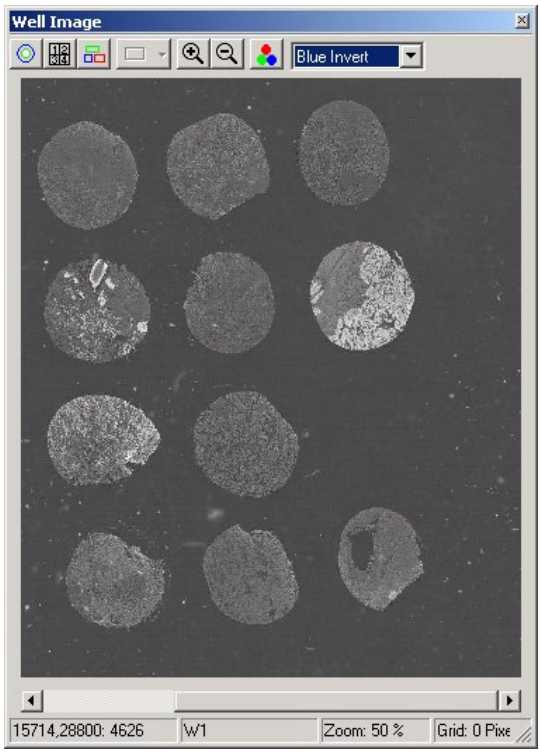
20. Select File and Save Workspace as: The save window will open providing you with an opportunity to name this workspace and any notations about the workspace. Name the workspace Tutorial Exercise 4.

Test Scanning

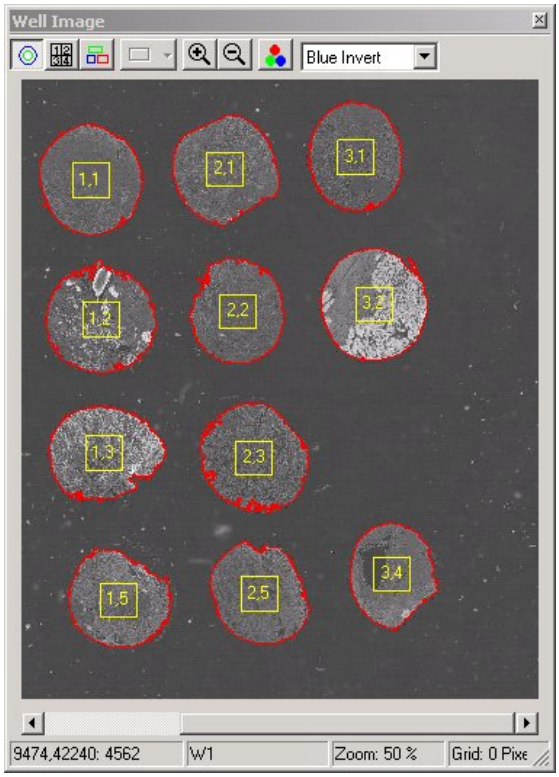
> Note: Although this tutorial will use a saved data file, steps 1-12 highlight how the raw data file was collected.

Low Resolution Scan

1. Select Field Image from the side Task bar, this will open the Field Image window This window is essentially the same as the Scan View window in software version 2.6, with one notable exception; the scan control keys have been removed from the Field Scan window and placed in the side Task Bar.
2. Place the micro array slide in an appropriate carrier and place the carrier on the instrument.
3. Move the stages so that the objective lens is under tissue micro-arrays. Open the laser shutter to use the laser as a pointer.
4. Open the Carrier window and set a scan area that is large enough to encompass all tissue sections in the array.
5. Perform a test scan of the tissue. Observe the scatter images in the Field View window.
6. Locate an area in which there are areas of blank space as well as tissue. Use the cursor to obtain the intensity level of the pixels in the blank area. Adjust the Scatter1 Gain setting so that the intensity of the pixels in this area is 14,000.
7. Stop the Test Scan. Open the Protocol Window and ensure the bridge between the Mosaic Scan and Field Scan modules reads “Pause”.
8. Initiate a Scan and Save.
9. At the completion of the low-resolution scan, the instrument will pause, open the Well image to display the Inverted Scatter tissues micro array images.



10. Click on the View Contour button to display the Tissue events (red) as well as the high-resolution scan areas (yellow).



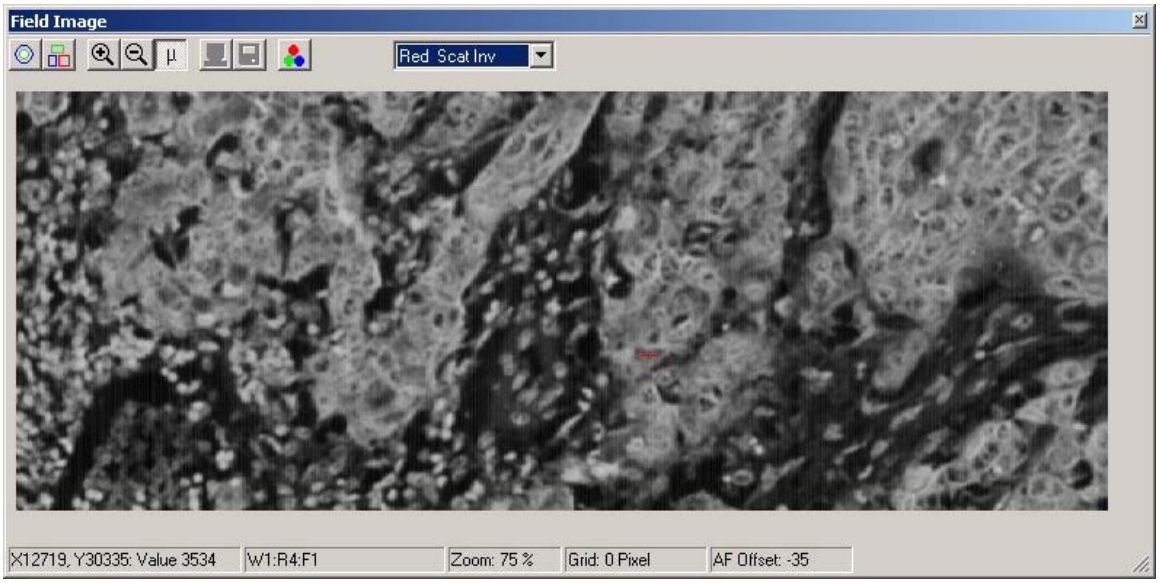
Note: It may be necessary to adjust the threshold value to properly contour on the tissue micro arrays. If this is the case, adjust the Threshold value in the first leg (low resolution scan) of the protocol window. To display the high-resolution scan area "rescan" the data file. This will be covered later in this tutorial.

12. Select Stop

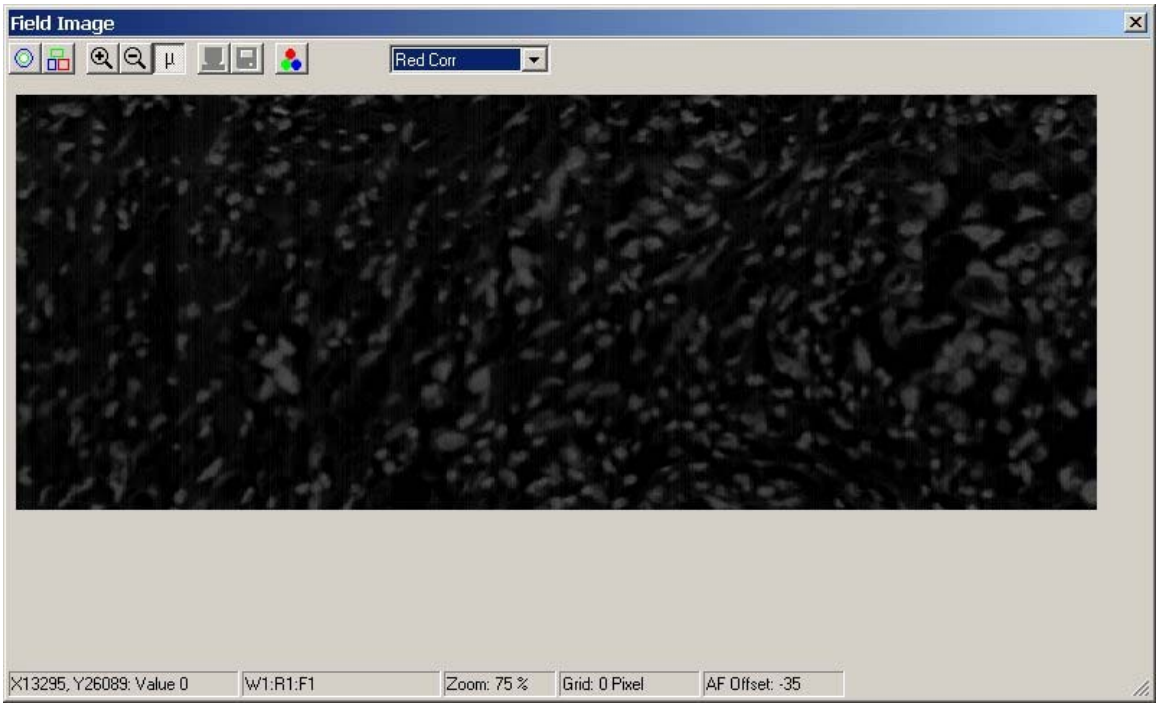
Adjusting the green PMT and Scatter2 channels for high resolution scan

1. Select **Open Run** and navigate to where the low resolution scan data file is located.
2. In the Analysis Mode section, highlight **Reanalyze scale 1 and scan scale 2** then click on **OK**.
3. Click on **Select Active Well** in the carrier window then click on the scan area. The scanned tissue micro arrays will be displayed along with the high resolution scan areas in the Well view window.
4. If necessary adjust the Threshold value in the low resolution scale and refresh.
5. Perform a test scan to recalculate the high-resolution scan areas.
6. Deselect the Pause that connects the Mosaic scan module to the Field Scan module, this will initiate the high-resolution scan.
7. The Green fluorescence PMT is set to give detectable signal in areas of the tissue but a low signal (less than 500) in blank areas of the slide.
8. The compensation factors (Blue lightloss M and Red lightloss M) were set to correct for spectral overlap of DAB into the Red Channel and Hematoxylin into the Blue Channel.

Inverted Red Light Loss Signal



Inverted Red Light Loss Signal corrected for DAB overlap



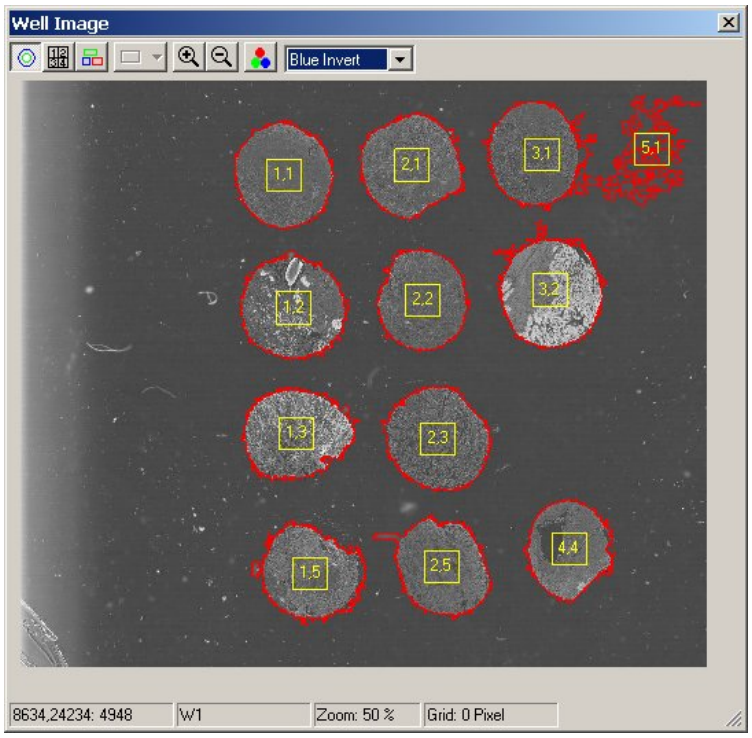
9. Once the photomultiplier tube and scatter detector settings have been established, the samples can be scanned saving the raw data files. Subsequent setting of segmentation and other analysis criteria can be done from the raw data files.

Opening Runs

Low Resolution Scan

For this part of the tutorial, insert the iCyte/iCys Revision 3.0 Tutorial data disk into the CD-ROM drive of the computer.

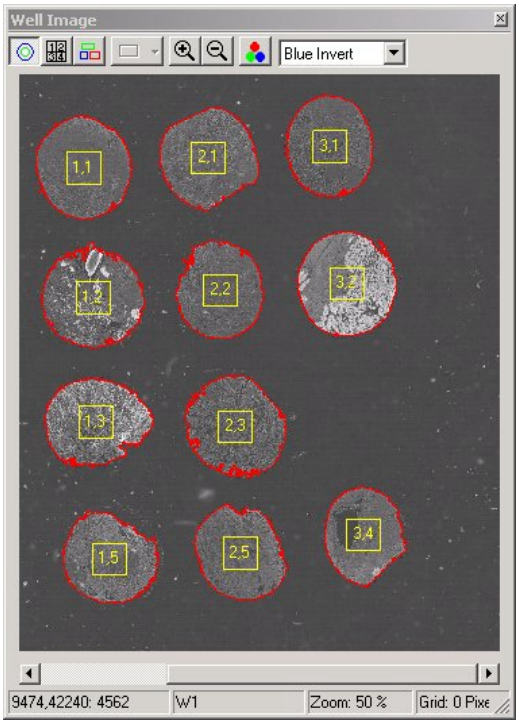
1. Select **Open Run** from the side Task bar, the Open Run window will be displayed
2. For this exercise click on the **Browse** button and navigate to the computers CD-ROM drive and select Tutorial Exercise 4 File 1.
3. Select the **Run. xml** file and **Open.**
4. Select **Reanalyze scale 1** as the Analysis Mode.
5. Select **OK**, the workspace selected will open.
6. Select **File > Open workspace** and select the Workspace that was just created.
7. Select **Test Scan** to start the reanalysis of the data file; the Well Image window will display the tissue micro arrays in red and the scan area's in yellow.



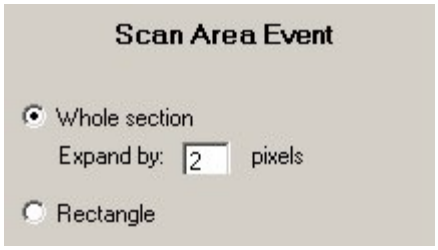
Notice that there is a scan area identified as 5,1. This is the result of the threshold being set too low. Before proceeding with the high-resolution scan this can be corrected.

8. Click on the Threshold Module in the Mosaic Scan leg of the Protocol window. The threshold value is set at 5000.
9. Use the slider to increase the threshold value to about 5500, and then click on the **Refresh** icon. The extraneous Tissue contour should no longer be seen.
10. Select **Test Scan** to reanalyze the data file, now the scan area 5,1 should also be cleared.

    Note: If this had been an actual low-resolution scan you would be able to reposition the scan area within the tissue micro arrays.
11. When the Threshold is adjusted the Well image window should look similar to the image below.



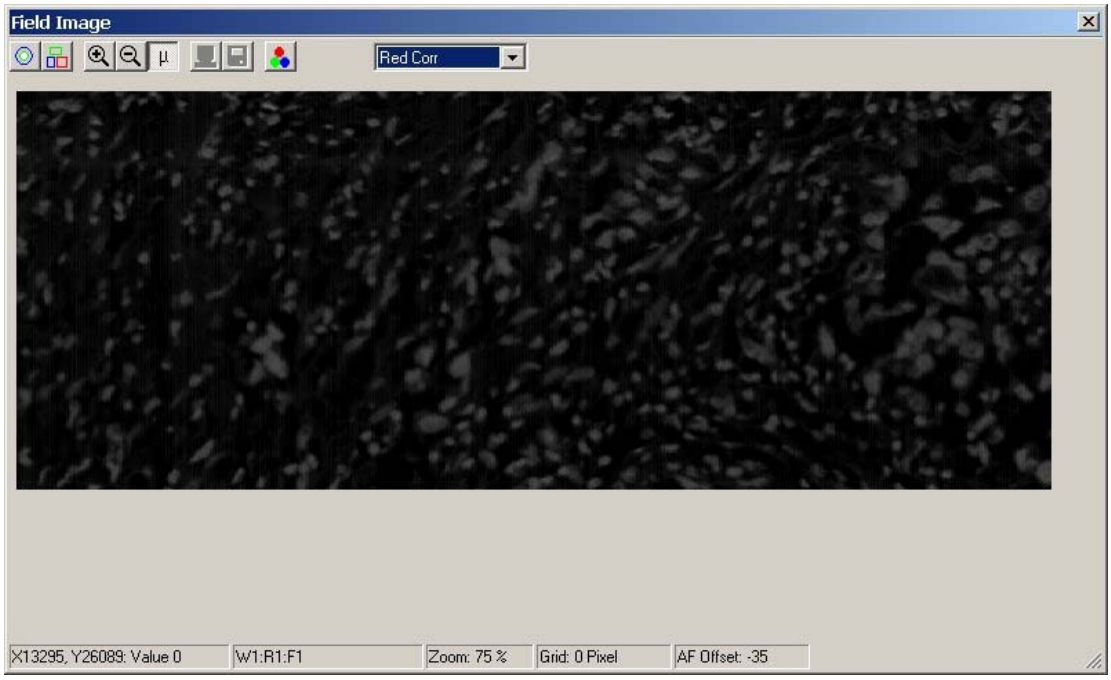
Note: In this example, a high-resolution scan area of 1000 microns x 1000 microns was defined in the Scan Area Event module. If the user wishes to perform a high-resolution scan on the entire tissue micro array select



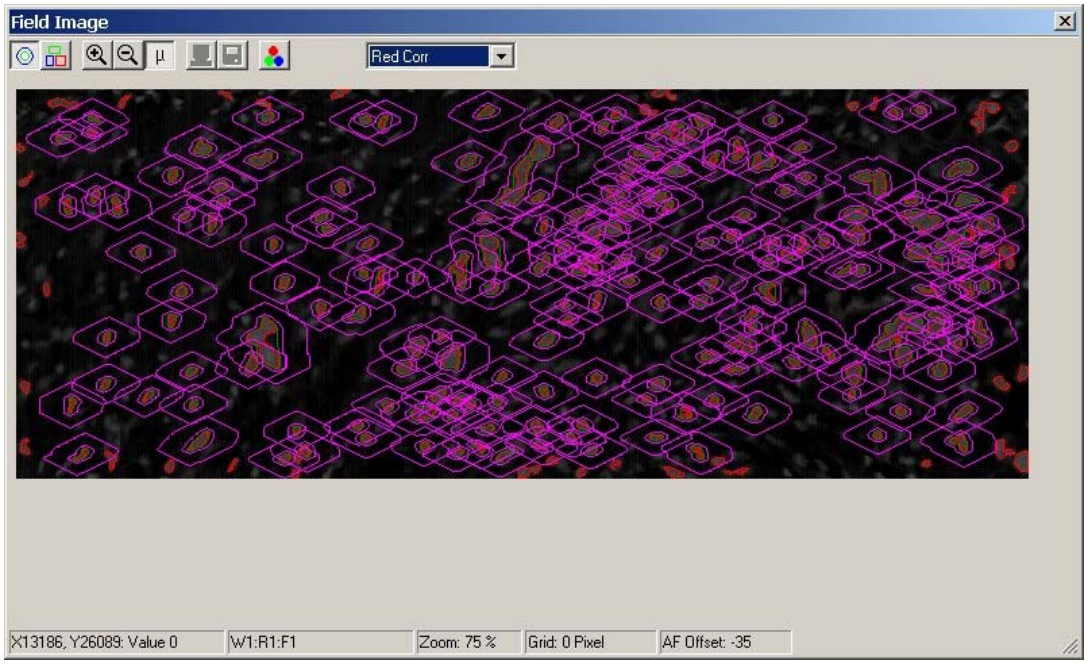
High Resolution Scan

1. Select **Open Run** from the side Task bar, the Open Run window will be displayed.
2. For this exercise click on the **Browse** button and navigate to the computers CD-ROM drive, Tutorial Exercise 4 File 2
3. Select Reanalyze scale 2 as the Analysis Mode.
4. Select **OK**, the workspace associated with the scan will open. Use this workspace.
5. If the Well Image window is not open, select **Well** View from the side Task Bar.

6. Click on the **Select Active Well** icon in the carrier window and click on the scan area of slide 1. This will display the tissue micro arrays and scan areas in the Well View window.
7. Click on **Single Field** in the scan control section of the side task bar to scan the first field.
8. View the Red Corr channel in the Field View window.

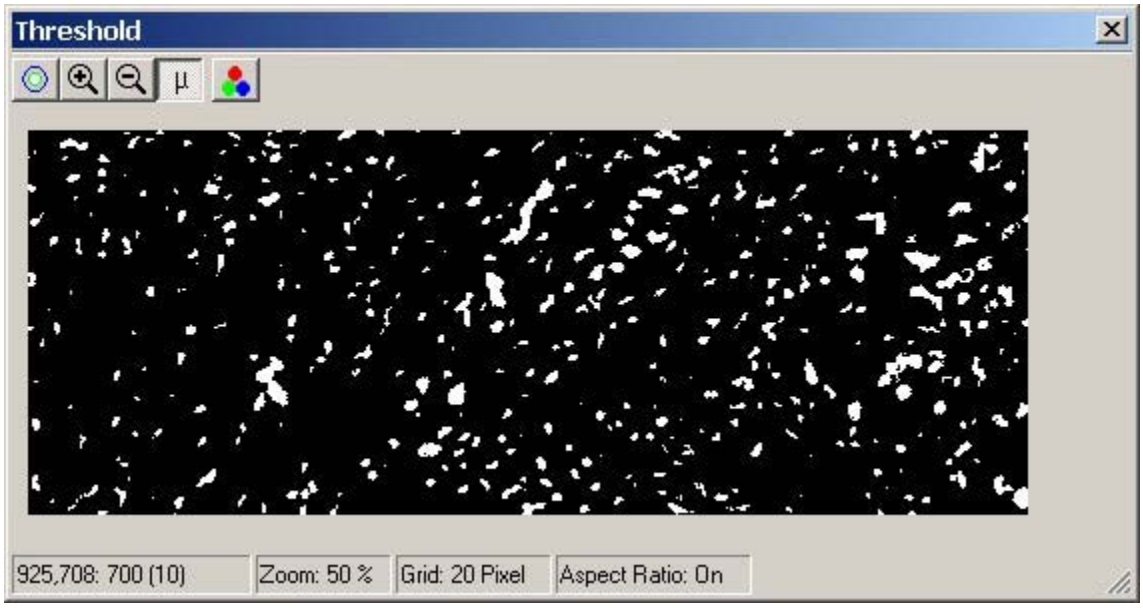


9. Select View Contours to view the cellular segmentation.

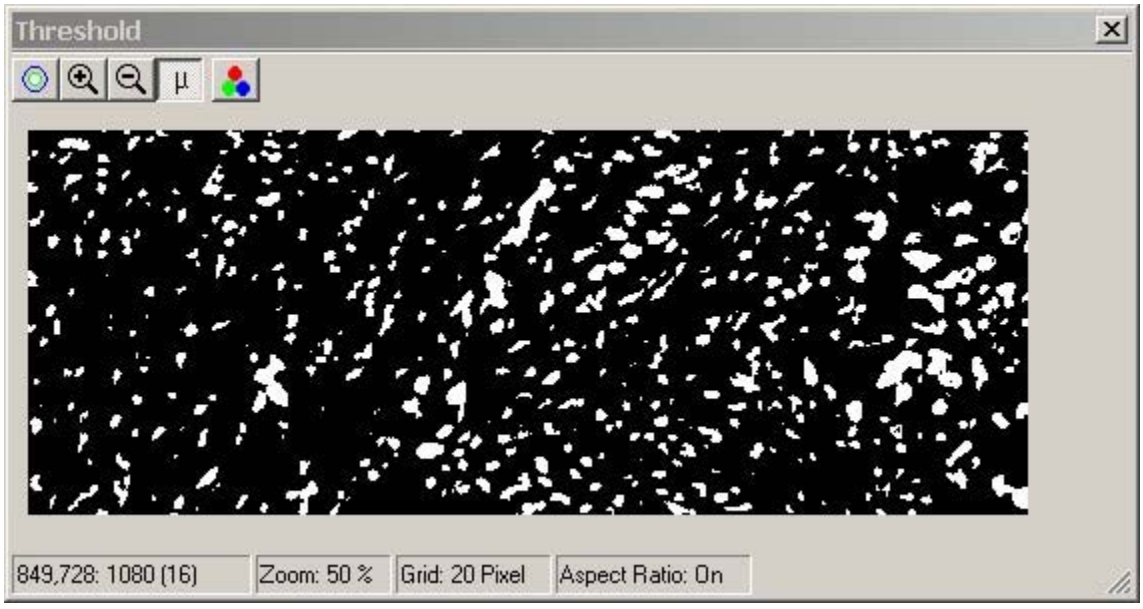


Note: Phantom Contour color has been set to none to clearly show event contours.

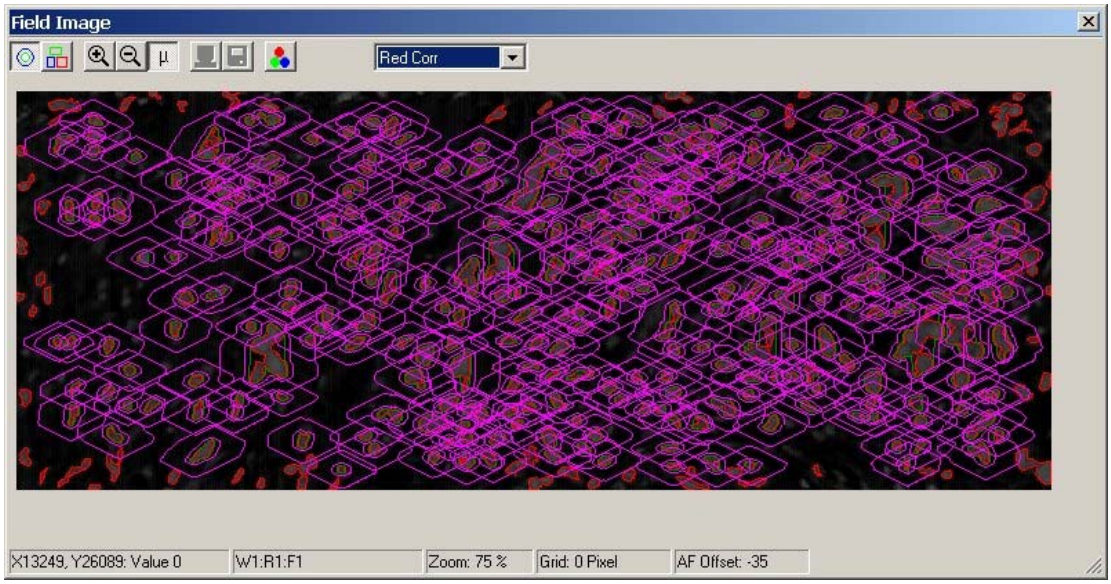
10. Open the Threshold module of the Field Scan scale (Protocol window).
11. Click on View Image icon to open the Interactive Threshold Window.



12. Adjust the slider in the Threshold module to change the threshold value. Moving the slider down (decreasing threshold) will allow for contouring of dimmer events but may lead to more multiple cell events.
13. Set the threshold value around 2300.



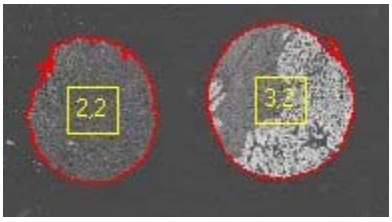
14. Click on the **Rescan** icon in the side task menu to see affect of the threshold in the Field View window.



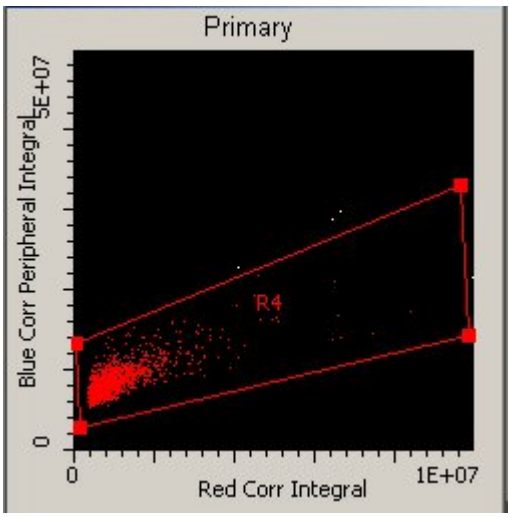
15. When you are satisfied with the cellular segmentation, stop the test scan.
16. Perform a Scan and Save.

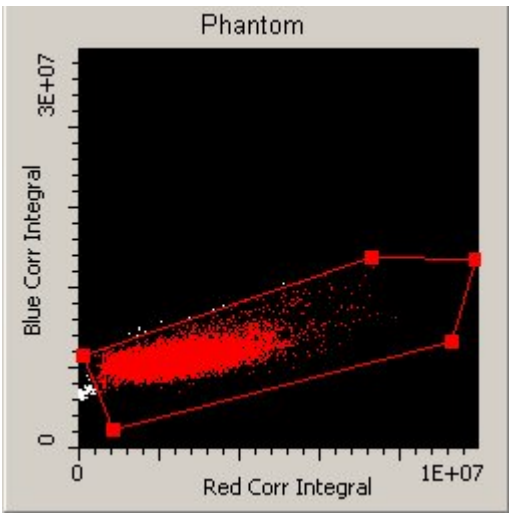
Analysis of Results

Areas positive for DAB staining will appear bright in the virtual channel Blue Invert in the Well Image window. Areas with no DAB staining will appear dark, an example of this is show below:

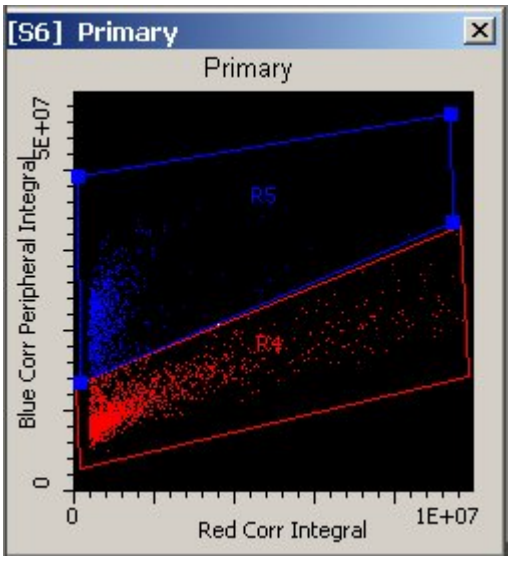


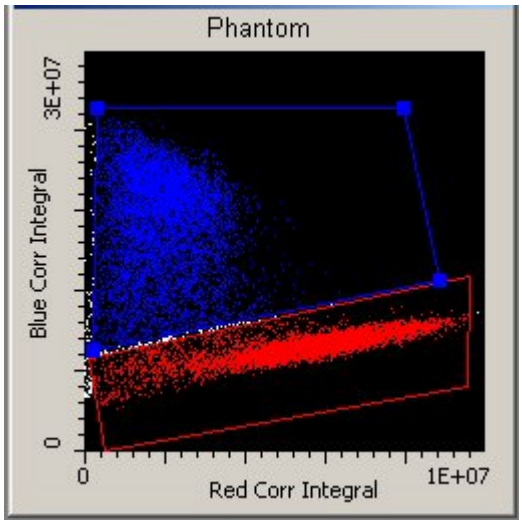
1. Open the Data Tab to display the event data from the scan.
2. Click on the Active well icon in the scan section of the side bar then click on scan area 2,2 these events are negative for DAB staining.
3. Draw a polygon around these events in the Phantom Red Corr Integral vs Blue Corr Integral and the Primary Red Corr Integral vs Blue Corr Peripheral Integral. Color these regions red.





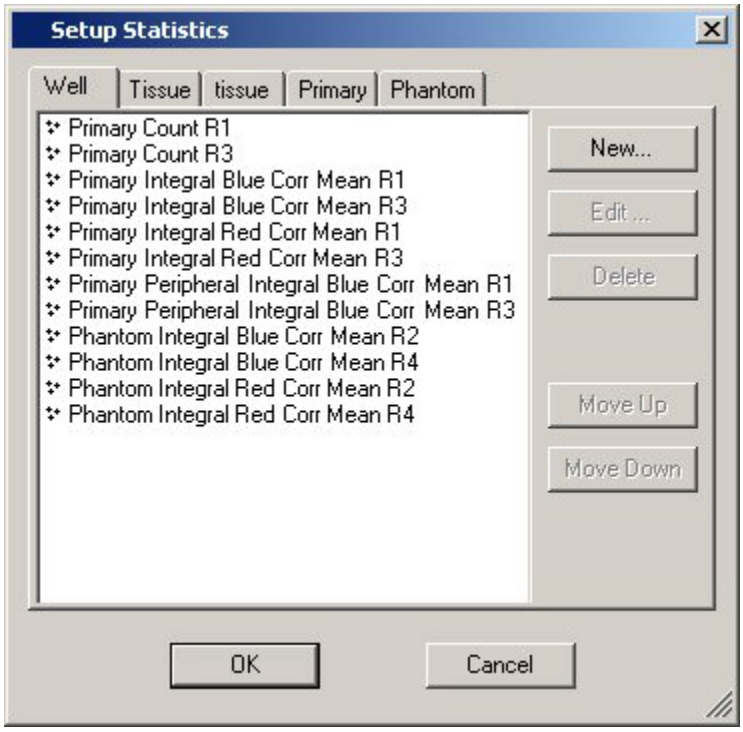
4. Highlight scan area 3,2, this is an array that is positive for DAB staining.
5. Draw a polygon around the events that do not fall into the region drawn earlier, color these region blue as shown below.





Run Statistics

1. Run statistics can be obtained for both the cellular segmented events and the phantom segmented events from the Statistics window accessed from the side task bar. Collection of these statistics allows for analysis of the data in iBrowser.


Setup Statistics
Well
Tissue
tissue
Primary
Phantom
Primary Count R1
Primary Count R3
Primary Integral Blue Corr Mean R1
Primary Integral Blue Corr Mean R3
Primary Integral Red Corr Mean R1
Primary Integral Red Corr Mean R3
Primary Peripheral Integral Blue Corr Mean R1
Primary Peripheral Integral Blue Corr Mean R3
Phantom Integral Blue Corr Mean R2
Phantom Integral Blue Corr Mean R4
Phantom Integral Red Corr Mean R2
Phantom Integral Red Corr Mean R4
New...
Edit ...
Delete
Move Up
Move Down
OK
Cancel